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The Ca^{2+}-binding protein parvalbumin: Molecular cloning and developmental regulation of mRNA abundance

(*in vitro* translation/cDNA library/oligonucleotide screening/muscle development/RNA blot analysis)

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ABSTRACT **Parvalbumin (PV) is a Ca^{2+}-binding protein found only in vertebrates. It is postulated to serve as a soluble relaxing factor in fast mammalian muscle. We have isolated a rat PV cDNA clone and used this as a probe to examine changes in PV mRNA during muscle and brain development. A cDNA library was constructed in PUC8/PUC9 plasmid vectors from adult poly(A)$^+$ RNA isolated from rat gastrocnemius muscle. The library was screened with a 17-mer oligonucleotide encoding amino acids 28–33 of rat PV. One recombinant (9f) was confirmed as a PV clone by DNA sequencing and was shown to contain 73% of the protein coding sequence. Hybridization of clone 9f to RNA separated by electrophoresis revealed two species 700 and 1100 nucleotides long but genomic blotting indicates that PV may be a single copy gene. Highest levels of PV mRNA are found in the gastrocnemius, which is a fast contracting/relaxing muscle. Skin contains the next highest amount of PV mRNA followed, in order, by brain and the slow twitch soleus muscle. Rat muscle PV mRNA levels increase 15- to 20-fold between postnatal days 4 and 20 as measured by dot blot hybridization of total RNA, whereas only a slight increase was observed when young and adult brains were compared. The changes in PV mRNA during development appear to be selective, because mRNA coding for the structurally homologous Ca^{2+}-binding protein calmodulin (CaM) was found to change only slightly in muscle. However, CaM mRNA levels decrease during the early days of brain ontogeny. Thus, the mRNAs that encode the homologous Ca^{2+}-binding proteins PV and CaM appear to be developmentally regulated in a tissue-specific manner.**

Parvalbumin (PV) belongs to a family of low-molecular-weight high affinity Ca^{2+}-binding proteins that are related by their homologous primary sequences (1, 2). In contrast to the multifunctional Ca^{2+} receptor calmodulin (CaM), which is present in all eukaryotic cells (3, 4), PV is only found in a limited number of vertebrate tissues where it is restricted to a few distinct cell types (5). In mammals, PV is most concentrated in fast-twitch muscle fibers and may be involved in the relaxation process (6, 7). Cross-innervation of fast skeletal muscle with a nerve normally connected to slow muscles leads to a considerable decrease of PV concentration, indicating that expression is dependent on neuromuscular interactions (8).

PV has also been purified from rat brain, but it is present in much lower quantity than in fast skeletal muscle (≈0.01% vs. 1.0% of the total protein, respectively) (9). Immunohistochemical methods have demonstrated that distribution of PV in brain is restricted to a subpopulation of neurons (10). However, neither the molecular basis nor the physiological significance of this cell-specific expression of PV is known.

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We have isolated PV cDNA clones from a cDNA library prepared from rat gastrocnemius muscle mRNA. Sequence analysis revealed one clone (9f) to contain 73% of the amino acid coding region. This clone has been used (*i*) to characterize mRNA species encoding PV, (*ii*) to hybridize to genomic DNA from different species, and (*iii*) to evaluate changes in the abundance of PV mRNA that occur during postnatal development of rat brain and skeletal muscle. These developmental changes have been compared to those of the mRNA for CaM (11), which is a structurally related Ca^{2+}-binding protein.

MATERIALS AND METHODS

All rats were Sprague–Dawley. Polyclonal antibodies against rat PV were raised in rabbits and affinity purified from antiserum as described (5). The synthetic 17-mer oligonucleotide mixture was obtained from Creative Biomolecules (San Francisco, CA). Avian myeloblastosis reverse transcriptase was from Life Sciences (St. Petersburg, FL). The Klenow fragment of *Escherichia coli* polymerase I was kindly provided by R. E. Moses (Baylor College of Medicine, Houston, TX). All other enzymes were from Bethesda Research Laboratories. Rabbit reticulocyte lysate was prepared according to the method of Crystal *et al.* (12).

mRNA Isolation, *in Vitro* Translation, and Immunoprecipitation. RNA was prepared from adult rat gastrocnemius muscle that had been placed in liquid nitrogen immediately after dissection. A phenol/$NaDodSO_4$ extraction method (13) followed by poly(A)$^+$ affinity chromatography (14) was used to obtain messenger RNA. One microgram of poly(A)$^+$ RNA was used for *in vitro* translation analysis in a rabbit reticulocyte lysate system (12). Translation was initiated by the addition of [^{35}S]methionine (125 μCi; 1 Ci = 37 GBq) and mRNA. After a 90-min incubation at 30°C, [^{35}S]methionine incorporation was measured by trichloroacetic acid precipitation and the protein products were analyzed by electrophoresis on 15% polyacrylamide gels containing $NaDodSO_4$. The gels were processed for fluorography using the method of Bonner and Laskey (15). For immunoprecipitation, 20 μl of the translation mixture was diluted with 80 μl of H_2O, incubated for 10 min at 90°C, and centrifuged. To the supernate a 20% $NaDodSO_4$ solution was added to give a final concentration of 1%. Four volumes of the following buffer was then added: 50 mM Tris·HCl, pH 7.4/190 mM NaCl/6 mM EDTA/2 mM EGTA/1.25% Triton X-100. Five microliters of anti-parvalbumin antiserum or 8 μg of affinity-purified antibody was added per 25 μl of the original translation mixture. The immunoreaction was allowed to take place overnight at 4°C on a tilting platform followed by incubation with protein A-Sepharose (Pharmacia) (30 μl of a 1:1 suspension in water per 25 μl of original translation mixture) for 3 hr at room temperature. After centrifugation in an Ep-

Abbreviations: PV, parvalbumin; CaM, calmodulin.

pendorf centrifuge, the pellet was washed three times at room temperature by shaking for 30 min with 1 ml of buffer. Three additional washes were done without Triton X-100. Forty-five microliters of gel sample buffer was added to the pellet and the mixture was heated for 5 min at 95°C. The protein A-Sepharose was collected by centrifugation and an aliquot of the supernatant fluid was counted in a liquid scintillation counter. The remainder was analyzed on 15% polyacrylamide gels containing $NaDodSO_4$ and processed for fluorography as described by Bonner and Laskey (15).

Preparation of a cDNA Library. The cloning strategy described by Helfman *et al.* (16) was used with minor modifications. Ten micrograms of poly(A)$^+$ RNA from gastrocnemius muscle was used as template for first strand cDNA synthesis by reverse transcriptase (17). The hairpin loop formed after alkaline hydrolysis of the mRNA was used to prime synthesis of the second strand of cDNA. This reaction was catalyzed by the Klenow fragment of *E. coli* DNA polymerase I for 15 hr at 15°C followed by 1 hr with reverse transcriptase at 37°C. *Eco*RI linkers were added to half of the preparation, the hairpin loop was cleaved with S1 nuclease, and then *Sal* I linkers were added. The sequence of linker addition was reversed for the other half of the double-stranded cDNA. cDNA was ligated in the former case to PUC8 and in the latter to PUC9 (18), both restricted with *Eco*RI and *Sal* I. The ratio of vector/cDNA ranged from 5 to 12.5. Transformation of *E. coli* JM 103 was carried out according to Hanahan (19). From 100 ng of cDNA, 30,000 recombinant clones were obtained. The clones were streaked on 30 nitrocellulose filters and replica-plated. The filters were either stored at −70°C, used for screening, or used as master plates.

Screening with Oligonucleotide and Identification of cDNA Clones. Bacterial recombinants on nitrocellulose filters were incubated on ampicillin plates (35 μg/ml) to achieve colonies of ≈1-mm diameter. The clones were allowed to grow for 2 hr with a Whatman 541 filter layered on top of the nitrocellulose filters. The colonies attached to the filters were transferred to a new ampicillin plate containing chloramphenicol (250 μg/ml), and plasmids were amplified for 20 hr. The filters were air-dried and treated with 0.5 M NaOH twice for 5 min each, 0.5 M Tris·HCl (pH 7.4) twice for 5 min each, 2× NaCl/Cit (1× NaCl/Cit = 0.15 M NaCl/0.015 M Na citrate, pH 7.0) twice for 5 min each, rinsed briefly with 95% ethanol twice and air-dried.

Prehybridization was carried out for 2 hr at 55°C in 0.9 M NaCl/0.09 M Tris·HCl, pH 7.5/0.006 M EDTA, containing 0.5% Nonidet P-40 and 250 μg of *E. coli* tRNA per ml. Hybridization was performed in the same solution containing the ^{32}P-labeled oligonucleotide mixture (4×10^6 cpm/pmol at 2 pmol/ml). The temperature for hybridization was 8°C below the calculated minimal melting temperature (t_m = 40°C) using the empirical formula t_m = 2°C (number of dA·dT base pairs) + 4°C (number of dG·dC base pairs) (20). After 20 hr of hybridization, the filters were washed at 27°C for 20 min, at 5°C for 15 min, and at 40°C for 45 min. They were then air-dried at room temperature and subjected to autoradiography. Positive clones were picked from master plates and again screened as described above. Plasmids were isolated as described by Katz *et al.* (21) and DNA sequencing was performed using the Maxam–Gilbert technique (22).

Blot Hybridization of RNA. Poly(A)$^+$ RNA from different rat tissues was isolated as described above. Ten micrograms of poly(A)$^+$ RNA was electrophoresed on gels containing 1.4% agarose and 6% formaldehyde and blotted onto Biodyne membranes (Pall, Glen Cove, NY) in 20× NaCl/Cit (23). Prehybridization, hybridization, and autoradiography were carried out as described in the manufacturer's manual.

Blot Hybridization of DNA. DNA from rat, mouse, and chicken was isolated by the method of Blin and Stafford (24). This high-molecular-weight DNA was digested to completion with *Eco*RI or *Bam*HI, electrophoresed on 0.7% agarose gels, and transferred to a Genescreen (New England Nuclear) filter by the method of Southern (25). For prehybridization and hybridization, the method of Karathanasis *et al.* (26) was followed.

Dot Blot Hybridization of RNA. Total RNA was prepared from brain and gastrocnemius muscle of rats of different ages (0–90 days postnatal) by homogenization in 4 M guanidine thiocyanate/0.1 M 2-mercaptoethanol (27). After centrifugation through a 5.7 M $CsCl_2$ cushion, the RNA-containing pellet was dissolved in H_2O and adjusted to 1 μg/μl. RNA was denatured by incubation in 50% formamide/6% formaldehyde/20 mM phosphate buffer (pH 6.5) for 5 min at 60°C. After quick cooling to 4°C, an equal volume of 20× NaCl/Cit was added (28). For the standard curve, rat gastrocnemius poly(A)$^+$ RNA was processed as described above. Dilutions were made in 10× NaCl/Cit/3% formaldehyde/20 mM phosphate buffer. All samples except for the standards contained 4.5 μg of RNA and were loaded in a volume of 180 μl. Results appeared to be more consistent if an equal volume of 10× NaCl/Cit was loaded prior to and after sample dotting. A Biodyne membrane (1.2-μm pore size) was used in a manifold apparatus and the flow rate was adjusted to allow one sample to be aspirated in 20–30 sec. The filters were processed according to the manufacturer's manual and 10^7 cpm of [^{32}P]cDNA per ml was used for hybridization. Fuji RX films were exposed at −70°C. The signals were quantitated by digital image processing using a 16-kilobyte image version of the gel analysis program of Mariash *et al.* (29).

Labeling of cDNA Probes. cDNA was labeled according to the random oligo-priming method of Feinberg and Vogelstein (30). Approximately 75% incorporation of the added αdCTP (0.05 mCi; 3000 Ci/mmol) was obtained using 20–30 ng of DNA. This resulted in a specific radioactivity of $2–3 \times 10^9$ dpm/μg. Prior to use, the probe was separated from free radioactivity by passage over a 1-ml Sephadex G-50 column in 20 mM NaCl/20 mM Tris·HCl (pH 7.5)/2 mM EDTA/0.25% $NaDodSO_4$.

RESULTS

***In Vitro* Parvalbumin Synthesis.** Poly(A)$^+$ RNA isolated from rat gastrocnemius muscle was assayed for PV mRNA by *in vitro* translation as shown in Fig. 1. As can be seen in lanes 2 and 3, actin (M_r, 42,000) is the major product synthesized from the total poly(A)$^+$ RNA. Heat treatment of the translation mixture (90°C, 10 min) eliminated 95% of all trichloroacetic acid-precipitable ^{35}S-labeled protein. PV was precipitated from the heat-stable translation products by an affinity-purified polyclonal antibody raised in rabbits (lane 4). Only one band was visible after electrophoresis and fluorography of the immunoprecipitate. Its relative molecular weight (M_r, 11,500) is identical to PV isolated from rat leg muscle (9) and labeled with [^{14}C]formaldehyde by reductive methylation (lane 1). Incorporation of ^{35}S immunoprecipitable PV represented 0.8%–1.4% of the total [^{35}S]methionine incorporated into protein.

Isolation of Recombinants from a cDNA Library and Identification as PV Clones. A synthetic 17-mer oligonucleotide containing a redundancy of 16 was synthesized that corresponded to amino acids 28–33 of the rat PV sequence (31) (Fig. 2). The oligonucleotide was labeled with ^{32}P and used to screen the gastrocnemius cDNA library. Four positive signals were found; three in PUC9 and one in PUC8. One of these recombinant clones, 9f, was chosen for further characterization by DNA sequencing and restriction enzyme analysis. The 240-base-pair insert was sequenced from the

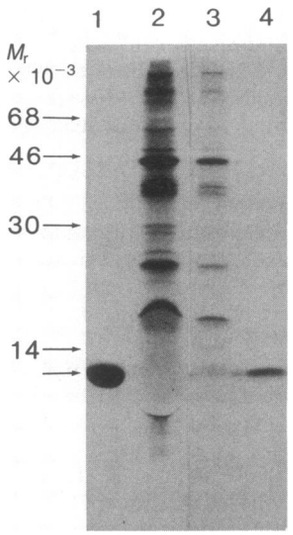

FIG. 1. *In vitro* translation and immunoprecipitation of PV. Translation of gastrocnemius muscle poly(A)$^+$ RNA was carried out in a reticulocyte system. Proteins were separated on NaDodSO$_4$/15% polyacrylamide gels; [^{35}S]-methionine-labeled proteins were detected by autoradiography. Lower arrow, position of authentic rat PV (M_r, 11,500). Lanes: 1, ^{14}C-labeled rat PV (10 μg, 80,000 cpm); 2 and 3, total translated proteins (4 and 1 μl of original translation mixture, respectively); 4, immunoprecipitation from heat-treated translation products (8 μl of original translation mixture) with rabbit anti-rat polyclonal antibody followed by incubation with protein A-Sepharose. DuPont Cronex x-ray film was exposed for 24 hr.

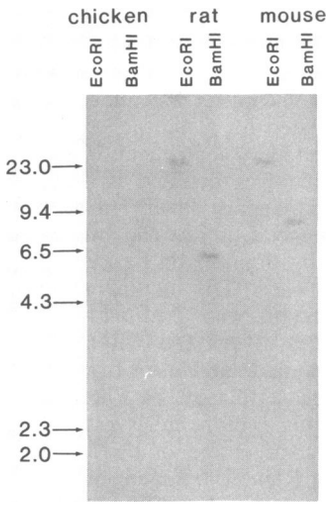

FIG. 3. Genomic DNA blot hybridized with PV clone 9f. DNA from chicken, rat, and mouse was digested with *Eco*RI or *Bam*HI as indicated. Seven micrograms of the reaction product was electrophoresed and blotted onto nitrocellulose after cleavage with 0.25 M HCl and denaturation with 0.5 M NaOH. The filter was hybridized with 9f (2 × 10^6 cpm/ml) under the following hybridization conditions: hybridization at 68°C in 6× NaCl/Cit, washing at 52°C in 2× NaCl/Cit for 3 hr. A Fuji RX film was exposed for 5 days with a Lightning Plus intensifier screen.

*Eco*RI and *Sal* I restriction sites toward the center. As shown in Fig. 2, translation of the 91 sequenced bases reveals complete identity with the amino acid sequence of rat PV (31). These data show that clone 9f contains only PV protein coding region, which extends from amino acid 22 to 102 (Fig. 2). A restriction enzyme map of clone 9f is also presented in Fig. 2. The *Eco*RI and *Sal* I sites shown designate the synthetic linkers used for cloning. One possible nucleotide sequence encoding amino acids 101 and 102 contains an *Eco*RI restriction site. It is therefore possible that the *Eco*RI site shown in Fig. 2 is not derived from the synthetic linker but is instead encoded by the cDNA. This is supported by the fact that all of the clones analyzed terminate in the same codon.

Analysis of Genomic DNA from Different Species. DNA from chicken, rat, and mouse was isolated and digested with the restriction endonucleases *Eco*RI and *Bam*HI, electrophoresed, blotted onto nitrocellulose, and analyzed using the labeled 9f insert as a hybridization probe (Fig. 3). Both rat and mouse DNA have a 25-kilobase *Eco*RI fragment containing PV sequences as well as a single *Bam*HI fragment that is 6 kilobases in rat and 8.5 kilobases in mouse. In the *Eco*RI digest of rat DNA, two fainter hybridization signals were also observed. Under the moderately stringent hybridization conditions used (hybridization at 68°C, 6× NaCl/Cit, washing at 52°C, 2× NaCl/Cit, 3 hr), no cross-hybridization of the rat probe with chicken DNA was observed. However, when the stringency was reduced (50°C for hybridization in 6× NaCl/Cit, washing at 50°C, 2× NaCl/Cit, 1 hr), signals could also be detected in the lanes containing chicken DNA (results not shown). The presence of only one major hybridization signal when *Eco*RI- or *Bam*HI-digested rat and mouse DNA were analyzed indicates that the PV gene may be single copy.

mRNA Analysis by Blot Hybridization. In all rat tissues examined, two mRNA species hybridized to the PV cDNA clone 9f. These bands are 1100 and 700 nucleotides long (Fig. 4). In all cases, the lower-molecular-weight band is present in higher abundance. By densitometric scanning of several RNA blots from different tissues, the ratio of the 1100- to the 700-base-pair band was always found to be ≈0.35. Adult gastrocnemius muscle contains the greatest amount of PV

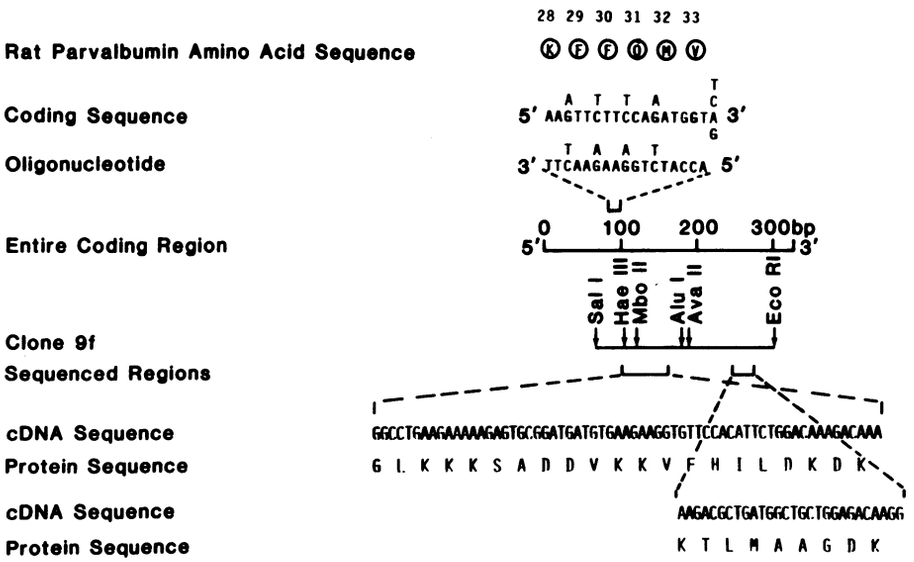

FIG. 2. Structure of synthetic oligonucleotide used for screening and partial sequence of PV clone 9f. The oligonucleotide mixture used was deduced from rat PV amino acids 28–33 as indicated. A partial restriction enzyme map is shown for PV clone 9f. The sequenced regions of this clone are indicated by brackets and cDNA sequence obtained is presented together with the corresponding amino acid sequence. The one-letter code for amino acids is as follows: A, alanine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; Q, glutamine; S, serine; T, threonine; V, valine.

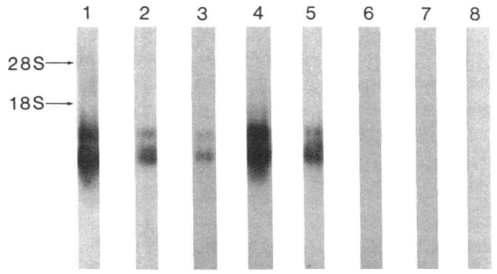

FIG. 4. RNA blot hybridization with PV clone 9f. RNA from different rat tissues was isolated by a $NaDodSO_4$/phenol method and affinity-purified on an oligo(dT) column. Poly(A)$^+$ RNA was electrophoresed on agarose gels (1.4%) in the presence of 6% formaldehyde and blotted onto a Biodyne-A membrane (1.2-μm pore size). Lanes: 1, adult gastrocnemius muscle, 1 μg; 2, 8- to 10-day-old gastrocnemius muscle, 2 μg; 3, adult soleus muscle, 10 μg; 4, adult skin, 10 μg; 5, adult brain, 10 μg; 6, adult heart, 10 μg; 7, adult liver, 10 μg; 8, adult kidney, 10 μg. A Fuji RX film was exposed with a Lightning Plus intensifying screen for 7 days.

mRNA (lane 1). The signal found in skin (lane 4) was considerably less intense, while RNA from the slow twitch soleus muscle (lane 3) and brain (lane 5) contained PV mRNA in even lower amounts. In contrast, no signal could be detected in RNA from heart (lane 6), liver (lane 7), or kidney (lane 8).

Developmental Regulation of PV Expression. To study the expression of PV mRNA during development, total RNA was extracted from leg muscles and brains of rats between the day of birth and 90 days postnatally. After denaturation in formamide/formaldehyde, the RNA was dotted on a Biodyne membrane and hybridized to clone 9f under stringent conditions. All samples contained 4.5 μg of total RNA from the indicated development stages (Fig. 5). To obtain a standard curve and to demonstrate the reproducibility of the dotting technique, poly(A)$^+$ RNA from adult gastrocnemius muscle was dotted in concentrations ranging from 16 to 500 ng. All samples, including standard poly(A)$^+$ RNA, were dotted in the same amounts on a second filter that was probed with a ^{32}P-labeled chicken CaM cDNA [350-base-pair *Rsa* I fragment from pCB12, containing only coding region (11)] under the same hybridization conditions.

Pronounced changes in the abundance of the PV mRNA during postnatal development of the gastrocnemius muscle are shown in Fig. 5. An increase was observed starting at day 5, and maximal values were achieved by day 20. Comparison of the mRNA content at day 20 to that at day 4 (before the increase begins) reveals a 15- to 20-fold difference. In contrast, the mRNA level in brain changes only slightly and reaches a plateau level at postnatal day 20. Brain mRNA levels are comparable to the amount found in muscle before the developmental increase. However, the signals in brain are above background at each age because no signal was found in liver at comparable RNA concentrations (data not shown). The increases in muscle PV mRNA are not due to alterations in the ratio of the 700- and 1100-nucleotide species. This ratio is 0.35 in poly(A)$^+$ RNA isolated from gastrocnemius muscle of 8- to 10-day-old rats (Fig. 4, lane 2) as well as in the adult (Fig. 4, lane 1).

CaM is known to be expressed constitutively (32) but is found in much higher levels in certain tissues of mammals such as brain and testes (33). Fig. 5 shows that CaM mRNA is expressed in much higher concentrations in brain than in muscle. In muscle, CaM mRNA increases only slightly during development, in marked contrast to the 15- to 20-fold difference found for PV. Similarly, little change in CaM mRNA was found to occur during brain development, and this alteration was a decrease during the early postnatal days.

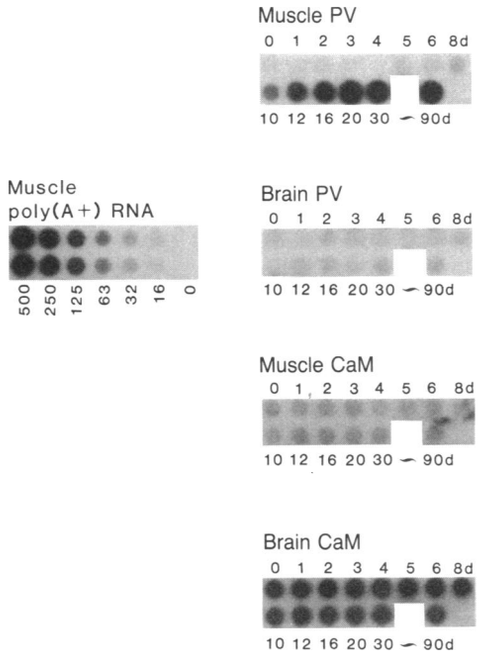

FIG. 5. Developmental changes in PV and CaM mRNA assessed by dot blot hybridization. Total RNA was prepared by guanidine isothiocyanate extraction and centrifugation through $CsCl_2$; 4.5 μg of total RNA was applied per dot. A Biodyne-A membrane (1.2-μm pore size) was used as support. Hybridization solution was applied at 10^7 cpm/ml. Adult gastrocnemius muscle poly(A)$^+$ RNA was dotted in nanogram concentrations as indicated and probed with the 9f PV cDNA insert to obtain a standard curve. RX Fuji films were exposed 5 days with a Lightning Plus intensifier screen.

DISCUSSION

Screening a gastrocnemius muscle cDNA library with a synthetic oligonucleotide that would encode amino acids 28–33 of rat PV resulted in four positive clones. Clone 9f was subjected to DNA sequence analysis and was shown to encode amino acids 22–102 of PV. Since rat PV contains 109 amino acids (31), this is 73% of the complete coding sequence. This clone produced hybridization signals with genomic DNA fragments from rat and mouse but not with chicken DNA, suggesting a much faster evolution of PV compared to the structurally homologous Ca^{2+}-binding protein CaM. In the latter case, chicken, human, and plant DNA reacted with a probe obtained from an *Electrophorus electricus* cDNA library (34). The presence of only one strongly hybridizing DNA fragment in both *Eco*RI and *Bam*HI endonuclease digests of mouse and rat DNA indicates that PV may be a single copy gene.

RNA blot hybridization revealed the presence of two PV mRNA species (700 and 1100 nucleotides long) in all tissues examined. The ratio of the signals seems to be similar in all RNA samples. It is possible that the two PV mRNAs result from differential polyadenylylation of a single primary transcript. Such a mechanism is responsible for generating multiple CaM (35) and dihydrofolate reductase (36) mRNA species. However, it is also possible that the 1100-nucleotide mRNA could be a precursor for the smaller mRNA.

The tissue specificity for PV expression recently demonstrated at the protein level (5) was confirmed here with respect to the presence of mRNA. The gastrocnemius muscle is mainly composed of fast anaerobic muscle fibers (type IIb) and contains the highest PV mRNA level. In contrast, the soleus muscle is another leg muscle that consists primarily of type I muscle fibers. It contains little mRNA that hybridizes to the PV cDNA. These data are compatible with the hypothesis that PV may function as a relaxing factor

in the process of fast muscle contraction/relaxation (7, 37). Finally, nonmuscle PV-containing tissues (5) (e.g., brain), were also shown to contain mRNA species that cross-hybridized to the PV cDNA, whereas those that do not contain PV (e.g., liver) produced no hybridization signals.

PV has been reported to be detectable in rat skeletal muscle by 4 days after birth (38). However, no developmental studies have previously been reported. We have demonstrated that PV mRNA is present in fast muscle from the day of birth and is developmentally regulated. An increase in the level of PV mRNA was observed starting at postnatal day 5. Parvalbumin mRNA continued to increase up to day 20, after which time it remained constant (days 30 and 90). The time period during which PV mRNA synthesis increases is paralleled by differentiation processes that lead to the formation of anaerobic fast contracting/relaxing muscle fibers in the gastrocnemius leg muscle. This differentiation process is believed to be under neural control. At the age of 20 days, when rats stop suckling and obtain full muscle speed, no further increase in PV mRNA levels was obtained. This developmental correlation between PV mRNA accumulation and anaerobic muscle function provides further support for its involvement in fast muscle Ca^{2+} transport mechanisms. In the brain, PV mRNA levels seem to be only slightly increased during development. The functional relevance of this finding cannot be addressed because no PV-dependent reactions have yet been described in the central nervous system.

CaM and its mRNA do not appear to be hormonally regulated (32) but have been shown to change during the cell cycle (39) and during development of the *Xenopus laevis* embryo (40). A slight increase in CaM mRNA was found during development of the gastrocnemius muscle, whereas a decrease was observed during the first few days of brain ontogeny. The latter case may reflect the fact that CaM levels are coupled to DNA synthesis (39) and cell division ceases early during development of the rat brain. At any rate, the small alterations in CaM mRNA are in marked contrast to the 15- to 20-fold increases in PV mRNA found during muscle development.

The availability of PV cDNA clones will allow further studies on the regulation of expression during cell-type transition as in the case of cross-innervation (8) or chronic stimulation of fast muscles (41) and also to provide answers concerning the function of PV in nonmuscle tissues. Furthermore, it will be of interest to study the relationship between the several homologous Ca^{2+}-binding protein genes that have been cloned. These genes include CaM (11, 35), myosin light chains (42), sea urchin Ca^{2+}-binding proteins (43), troponin C (44, 45), vitamin D-induced Ca^{2+}-binding protein (46, 47), and S-100 protein (48), all of which are believed to have a common evolution based on their homologous protein structure.

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